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The human estrogen receptor (hER) is a therapeutic target in estrogen-responsive breast cancers. It has been shown that a 12 amino acid phosphotyrosyl peptide composed of the sequence surrounding tyrosine 537 of hER is an inhibitor of the hER function. Base on a hypothesis that hER contains a discreet binding domain for the phosphopeptide, this project proposes to determine the three-dimensional structure of the proposed domain.

Based on previous binding assay data, we prepared a fragment of hER corresponding to residues 253-341. Characterization using fluorescence and nuclear magnetic resonance spectroscopy, we show that this hER fragment does not have a well-defined structure, even in the presence of the phosphopeptide. These results, together with recently determined crystal structures of the hER ligand binding domain and other mutation studies, indicate that the original hypothesis is incorrect. Our new experiments suggest that the phosphopeptide inhibits hER by binding to the ligand binding domain. Thus, the focus of this project has been shifted to identifying the binding site within the hER ligand binding domain and identifying critical interactions. Results from this project will provide insights into development of new hER inhibitors for treatment of estrogen-responsive breast cancers.

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Stephen Kovacic 8/17/98

PI - Signature Date

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INTRODUCTION

The estrogen receptor is a member of the steroid/thyroid hormone receptor superfamily. The human estrogen receptor (hER) serves as both a prognostic indicator and a therapeutic target in breast cancer. High levels of hER are seen half of all breast cancers and a third of these patients respond to hormone therapy. Though estrogen antagonists such as tamoxifen are effective treatments of breast cancers, an increase concern is the occurrence of tamoxifen-stimulated tumor growth. Further, some hER positive breast cancers have been reported to be non-responsive to hormone therapy.

The mechanism by which hER modulates transcription is still under intense investigation. However, it has been conclusively shown that only the dimeric form of hER is capable of binding to the estrogen response element (ERE) and transcription activation (Kumar & Chambon, 1988). Thus, agents that disrupt the hER homodimer independently of the receptor's hormone binding would be a new class of anti-hER therapeutics. Such agents may provide effective means to treat hER positive breast cancers that are non-responsive to hormone therapy.

Dr. Angelo Notides, the original PI of this project, who passed away shortly before start of this project period, extensively studied phosphorylation of hER (Arnold et al., 1994; Arnold et al., 1995a; Arnold et al., 1995b; Arnold et al., 1995c). Arnold and Notides reported that tyrosine (Tyr) 537 of hER is phosphorylated (Arnold et al., 1995c). Further, they found that a short peptide, CNVVPL(Yp)DLLLE, termed Yp537, consisting of the amino acid residues from 532-542 of hER including the phosphotyrosine 537, is capable of inhibiting the DNA binding function of hER (Arnold & Notides, 1995). The inhibition of hER by the peptide occurs irrespective of whether hER is unliganded or liganded with agonist or antagonist. These observations lead them to propose that hER contained a phosphotyrosine-binding domain, analogous to the src homology 2 (SH2) domain (Arnold & Notides, 1995). According to this hypothesis, phosphotyrosine 537 and the proposed phosphotyrosine-binding domain are involved in the hER dimerization through reciprocal coupling, as seen in another SH2 domain (Shuai et al., 1994).

Yudt and Notides then identified a fragment of hER that has an affinity to the Yp537 peptide by far Western blot analysis (unpublished; personal communication). Various hER fragments were expressed in *Escherichia coli* (*E. coli*) as glutathione-S-transferace (GST) fusion proteins, separated by electrophoresis, and immobilized on a membrane. The Yp537 peptide was ^{32}P-labeled and its binding to hER fragments was examined. These experiments showed that a 113 amino acid fragment corresponding to residues 253-365 was required for the binding of Yp537.

Primary purpose of this project is to determine the solution structure and ligand interaction of the proposed phosphotyrosine binding (PTB) domain of hER by means of state-of-the-art multi-dimensional heteronuclear NMR methods. The fundamental premise of this research proposal is that knowledge of the three-dimensional structure of the PTB domain of hER will lead to a new understanding of the underlying molecular mechanism involved in binding of the phosphotyrosine peptide to hER which inhibits the hER activity. This structural knowledge will form the basis for structure-based design of novel anti-estrogen agents that mitigate the action of hER.

The studies proposed in this project are based on two hypotheses. First, a phosphopeptide corresponding to the sequence surrounding phosphotyrosine 537 of hER can specifically disrupt interactions between phosphotyrosine 537 and a PTB domain of hER. Second, therapeutic agents that disrupt hER dimerization can be rationally designed based on the three-dimensional structure of the proposed hER PTB domain.

The proposed project aims to determine the 3D structure of the hER PTB domain in the complex with the phosphotyrosine-containing peptide. Our specific aims are:

1. to determine the minimal size of the hER PTB domain which is required for the PTB activity.
2. to prepare large quantities of the PTB domain for structural studies.
3. to determine the 3D structure of the PTB domain using nuclear magnetic resonance spectroscopy.
4. to identify critical molecular interactions responsible for the phosphotyrosine binding.

Statement of work in the original proposal is as follows:

Task 1:	Months 1-4; establish the minimal size of the PTB domain.
Task 2:	Months 5-14; determine optimum expression system and sample preparation methods
Task 3:	Months 12-16; establish NMR measurement conditions.
Task 4:	Months 16-20; collect a series of multi-dimensional NMR spectra.
Task 5:	Months 18-20; complete sequence specific resonance assignments.
Task 6:	Months 20-22; assign NOESY spectra and obtain distance constraints.
Task 7:	Months 22-24; calculate a low-resolution structure.
Task 8:	Months 24-30; refine the structure by obtaining additional constraints.
Task 9:	Months 24-36; site-directed mutagenesis of the PTB domain and functional and structural analysis of mutants.

BODY

Specific Aim 1 and 2 : identification of the PTB domain location (Task 1), and preparation of the PTB domain protein (Tasks 2 and 3)

Yudt and Notides (unpublished data; personal communication) have demonstrated using the far-Western binding assay that two fragments of hER retain the PTB binding activity. They are: an N-terminal fragment (amino acid residue 1-341) and a fragment corresponding to exon 4 (residues 253-365). The results suggest that residues 253-341 which are included in both fragment correspond to the smallest hER fragment as yet to be identified associated with the PTB activity. hER, like other members of the nuclear hormone receptor superfamily, has a modular architecture. The DNA binding domain and the ligand binding domain (LBD) are structurally well defined. The fragment starts just after the DNA binding domain of hER and ends just before the putative core of the ligand binding domain of hER. At that time the crystal structure of hER had not been determined, and the location of hER LBD was based on the sequence homology to ligand binding domains of other nuclear hormone receptor with known structure (Wurtz et al., 1996). It should be noted that the size of the fragment, 88 residues, is comparable to those of the SH2 domain (~95 residues) which specifically binds phosphotyrosine containing peptides. In addition, the fragment is well within the current size limit of NMR spectroscopy (~30 kDa) even when complexed with the target peptide (12 residues). Therefore, we chose to characterize the activity and physical properties of a 88 residue fragment.

RESULTS AND DISCUSSION

Expression and purification of hER[253-341]

Using an *E. coli* expression vector, we successfully overexpressed hER[253-341] as a fusion protein with an N-terminal poly-histidine tag (Figure 1A). A predominant majority of the protein was expressed as an insoluble form, presumably forming inclusion bodies (Figure 1B). Thus, we purified the protein by first solubilizing it by urea and then using a metal affinity chromatography. A highly pure sample could be prepared by these procedures (Figure 1c).

Characterization of hER[253-341]

Because hER[253-341] was purified as an unfolded form in urea, it needed to be refolded into an active form. The protein was found to be prone to aggregation when subjected to refolding conditions, i.e. low urea concentration. We screened different buffer conditions (pH, salt type and concentration, and temperature) for refolding. However, we were unable to find good conditions in which hER[253-341] is soluble up to a concentration required for NMR analysis (> 1 mM). Thus, we first characterized the protein using fluorescence spectroscopy which requires much lower protein concentrations.

hER[253-341] contains a single tryptophan residue at position 292. This tryptophan was used as a spectral probe to monitor conformational changes of the protein. The tryptophan side chain is highly hydrophobic and it is usually buried inside in a folded protein. Burial of the tryptophan side chain causes a large shift in the peak wavelength of the tryptophan fluorescence emission spectrum. We found that, under all conditions tested, no significant changes in the peak wavelength occurred when the protein was transferred from a high urea solution to a low urea solution (Figure 2). Also no significant change was observed when the phosphopeptide was added to the solution (Figure 2). These results suggest either that the environment of the tryptophan side chain does not change significantly upon refolding of hER[253-341], or that the protein is not refolded under these conditions tested.

In order to obtain conclusive data as to whether hER[253-341] is folded in urea-free solutions, we then characterize the protein using NMR spectroscopy. After screening many solution conditions, we discovered that the protein is soluble up to ~ 0.1 mM in sodium phosphate buffer (pH 6.5). This was the best among tested buffers (Tris-HCl, sodium phosphate and sodium acetate) and pH (4-8.5). We prepared a ^{15}N-labeled hER[253-341] sample, and collected ^{1}H, ^{15}N-heteronuclear single-quantum correlation spectra in the absence and presence of the phosphopeptide (Figure 3). An HSQC spectrum shows cross peaks between directly-attached amide ^{1}H and ^{15}N nuclei, thereby providing the fingerprint of a protein. In the absence of the target peptide, the HSQC spectrum of the protein (Figure 3a) has features characteristic of a random coil peptide. The ^{1}H chemical shifts are clustered around 8.2 ppm, and the cross peaks are highly degenerate. In addition, cross peaks for side chain NH_2 groups are also clustered near ^{15}N=113 ppm and ^{1}H=6.8 and 7.5 ppm. Then the phosphopeptide was added to the protein sample at a final concentration of 0.3 mM. Note that the HSQC experiment only detects ^{15}N labeled compounds, and thus the experiment can detect conformational changes in the ^{15}N labeled protein without interference from the added peptide. A few examples of ligand-induced folding have been reported (Zhang & Forman-Kay, 1995). The HSQC spectrum of hER[253-341] with the peptide shows very few changes from the spectrum of the peptide-free protein, indicating that there is no major conformational change upon the addition of the peptide, and that the protein

remains unfolded. Therefore, we concluded that hER[253-341] is unstructured and it is not capable of binding to the phosphopeptide under conditions tested.

Re-examination of original data supporting the location of a phosphotyrosine binding domain

The results from structural characterization of hER[253-341] described above prompted us to reexamine data supported the identity of the binding domain. The location was identified by far-western blotting experiments. In these experiments, various fragments of hER fused to GST were expressed in *E. coli*, and they were then separated on SDS PAGE, and then transferred to a blotting membrane. The membrane was probed with a ^{32}P-labeled phosphotyrosine peptide, and the bound peptide was detected by autoradiography. Thus, the hER fragments were once denatured in SDS, and the folding state of these proteins on the membrane was unknown.

In order to test whether these hER fragments interact with the phosphopeptide in aqueous solution, we, in collaboration with Matthew Yudt, examined binding of GST-hER fusion proteins to the phosphopeptide immobilized to agarose beads. Results indicate that hER[253-365] (exon 4) binds to the peptide-coupled beads as well as to the control, peptide-free beads. Fragments corresponding to exon 1 and exons 1 and 2, respectively, did not show significant binding to the peptide-beads. These observations suggest that the exon 4 fragment is highly adhesive. In addition, the exon 4 fragment (shown below) contains a large number of positively charged residue (shown in capital letters) which may be responsible for nonspecific interactions with the negatively-charged phosphopeptide.

```
253    giRKdRR ggRmlKHKRq RddgegRgev gsagdmRaan lwpsplmiKR
301 sKKnslalsl tadqmvsall daeppilyse ydptRpfsea smmgllltnla dRelvHminw aKRvp
```

In addition, other groups reported that mutations at Tyr537 does not abolish the transcription activity of hER. Interestingly, some of mutations at position 537 have been shown to be constitutively active (Weis et al., 1996; White et al., 1997; Zhang et al., 1997). These results appear to contradict with those of Arnold et al. (Arnold et al., 1995c) showing Tyr537 to Phe mutant was inactive. Recently, Daria Vorojeikina and Matthew Yudt in the (former) Notides laboratory demonstrated that the Tyr537Phe mutant is active but has a reduced stability (unpublished; personal communication). This reduced stability may have misled Arnold et al. to reach the above conclusion. Taken together, available data both from the Notides laboratory and from others indicate that phosphorylation of Tyr537 is not essential for the activity and dimerization of hER.

Analysis of hER LBD crystal structures

In the last nine months, two independent groups reported the crystal structures of the ligand binding domain (LBD) of hER (Brzozowski et al., 1997; Tanenbaum et al., 1998). These structures show that hER-LBD has a structure very similar to the LBD's of other nuclear hormone receptors (Bourguet et al., 1995; Renaud et al., 1995; Wagner et al., 1995). Because the structural coordinates have not been made available to us, we performed homology modeling using coordinates of RXR-LBD and a sequence alignment (Wurtz et al., 1996) (unpublished data). hER-LBD starts at residue 309 and ends at residue 546. The crystal structure of the DNA binding domain (DBD) of hER (Schwabe et al., 1993) showed that the core of DBD ends at residue 241. Taken together, these crystal structures suggest that the section of exon 4 that is not included in DBD or LDB (residues 243-308) is too small to constitute a discreet phosphotyrosine domain.

The two hER-LBD structures (Brzozowski et al., 1997; Tanenbaum et al., 1998) are very similar except for the position of helix 12. In one structure, helix 12 is extended to make contact with a neighboring molecule in the crystal (Tanenbaum et al., 1998) similar to apo RXR-LBD. In the other, helix 12 is folded back to the core of LBD similar to holo RAR-LBD. Interestingly, the position of helix 12 in the hER LBD-estradiol complex is quite different from that in the hER-raloxifene (antagonist) complex (Brzozowski et al., 1997). The large conformational change of helix 12 in these structures is consistent with the observation that helix 12 is essential for the function of transactivation function (Danielian et al., 1992). It should be noted that our phosphopeptide correspond to a C-terminal part of the loop connecting helices 11 and 12 and an N-terminal part of helix 12.

Possible mechanisms of the inhibitory activity of the peptides

The data described above collectively indicate that our original hypothesis that hER contains a discreet phosphotyrosine binding domain is not correct, forcing us to revise our working hypothesis. Matthew Yudt, in collaboration with us, has performed a number of experiments testing various possibilities of the inhibition mechanism of the phosphopeptide. Results of his experiments are summarized as follows (unpublished data; personal communication):

1. The phosphopeptide does not work by making a disulfide bond with hER.
2. The peptide inhibits the DNA-binding activity of hER in a non-competitive fashion, indicating that the peptide does not directly inhibit the interaction of hER DBD and its target DNA.
3. The peptide does not compete with hormone binding of hER.
4. The peptide does not inactivate hER by enhancing precipitation or degradation.

Very recently, Yudt demonstrated, using in vitro translated fragments of hER, that the phosphopeptide does not inhibit the DNA binding activity of an hER fragment corresponding to residues 1-362, but it does inhibit DNA binding of a fragment corresponding to residues 150-595 as well as full-length hER (unpublished data; personal communication). Both fragments contain the DNA binding domain and exon 4. These results indicate that the inhibition by the phosphopeptide requires LBD.

Though present data do not identify the site of the phosphopeptide binding, our current working hypotheses assume that the peptide binds to LBD. If our assumption that the peptide binds to LBD is correct, how would the peptide inhibit the DNA binding function of hER? Our hypotheses include the following:

1. The peptide was derived from the helix 12 region which undergoes a large conformational change upon hormone binding. The peptide may compete with this region, and induces a conformational change that inactivate the DNA binding activity.
2. The peptide, CNVVPLYDLLLE, has a homology to the helix 11, RLAQLLLILS... Helix 11 consist a large portion of the dimer interface between the LBD dimer. The peptide may bind to the dimer interface and inhibit the dimerization of hER. Inhibition of enzyme activity by such "interface peptides" has been demonstrated in other enzymes (Schramm et al., 1996; Prasanna et al., 1998).

These possibilities are currently being investigated in our laboratory.

Specific aims 3 and 4. Structure determination of hER phosphotyrosine binding domain, and identification of critical molecular interactions.

As described in detail above, our results have negated our original hypotheses on which these specific aims were based. Thus, specific aims 3 and 4 cannot be pursued as originally planned. However, we have identified that LBD is required for the inhibitory activity of the peptide. We propose that specific aims 3 and 4 be revised as (a) identification of the peptide binding site in hER LBD, and (b) identification of critical interactions between the peptide and its binding site.

CONCLUSIONS

We have demonstrated that our original hypotheses that hER contains a discreet phosphotyrosine binding domain, and subsequently that this domain lies in the hinge region are incorrect. Therefore, the original project, which was based on these hypotheses, could not be executed as proposed. However, the phosphopeptide does inhibit the DNA-binding activity of hER. Further, our data suggest that the phosphopeptide inhibits the hER activity by binding to the ligand binding domain of hER. Thus, we have revised our working hypotheses, and we are currently focusing on deciphering the action mechanism of the phosphopeptide inhibition. We believe that results from our investigation will provide important information for designing a novel anti-estrogen therapeutics.

EXPERIMENTAL PROCEDURES

Cloning and protein expression

The gene fragment corresponding to residues 253-341 of hER was cloned into *E. coli* expression vectors using standard polymerase chain reaction (PCR) protocols. Plasmid pET15b (Novagen) was used been used to express the fragment as a fusion protein with poly-histidine tag. In our experience, the system usually express foreign protein at a high level and hence suitable for preparing isotopically enriched samples. E. coli BL21(DE3) (Novagen) was transformed with the pET15b-hER[253-341]vector and the cells were grown in the M9 minimal medium supplemented with Bactotryptone (Studier et al., 1990). ^{15}N-enriched samples were made by expressing the protein in the M9 minimal medium supplemented with ^{15}N ammonium chloride as the sole nitrogen source. Protein expression was initiated by an addition of IPTG to the culture at a final concentration of 0.1-1.0 mM. We found that the hER fragment was not expressed well in the E. coli cells. We then found that we could increase the expression level of the hER fragment by using BL21(DE3) pLys cells (Novagen). These observations suggest that the hER fragment is mildly toxic to the cells.

Protein purification

The expressed protein was purified as follows. *E. coli* cells were suspended in 50 mM Tris HCl buffer (pH 8.0) containing 100 mM NaCl. The suspension was frozen and thawed. To the suspension, PMSF was added to a final concentration of 1 mM. The suspension was sonicated to disrupt chromosomal DNA. The solution was then centrifuged at 10 krpm in a SS-34 rotor

(Sorvall). The pellet was resuspended in the sample buffer containing 1 mM PMSF, and centrifuged as described above. The pellet was then solubilized in the buffer containing 6 M urea and 0.5 M NaCl.

The solubilized protein solution was applied to a nickel affinity column (Chelating Sepharose; Pharmacia) equilibrated in the buffer containing urea and NaCl. After washing the column with this buffer, the protein was eluted with the urea-containing buffer with 100-200 mM of imidazole. When necessary, the buffer of protein samples was exchanged to an appropriate buffer using a Centricon concentrator (Milipore).

Fluorescence spectroscopy

Fluorescence spectra were collected on Spectronic AB-2 and Photon Technology International spectrometers, both equipped with temperature control. Typically, emission spectra from 300 to 400 nm with the excitation wavelength at 290 nm were recorded on a sample containing 1-5 μM protein. The sample temperature was set at 25 °C.

NMR spectroscopy

All spectra were recorded on a Varian Unity INOVA 600 spectrometer. The probe temperature was set at 25 C. ^{1}H, ^{15}N-HSQC experiment was performed using published schemes (Kay et al., 1992; Grzesiek & Bax, 1993). Data were processed using the NMRPipe program (Delaglio et al., 1995) on a Silicon Graphics Unix workstation.

Molecular modeling

Homology modeling of hER LBD was performed using the protein design module in the program Quanta (Molecular Simulations). The structural coordinates for RXR-LBD (Protein Data Bank entry 1LBD) was used as the starting structure. The modeled structure was then energy minimized using CHARMm (Molecular Simulations).

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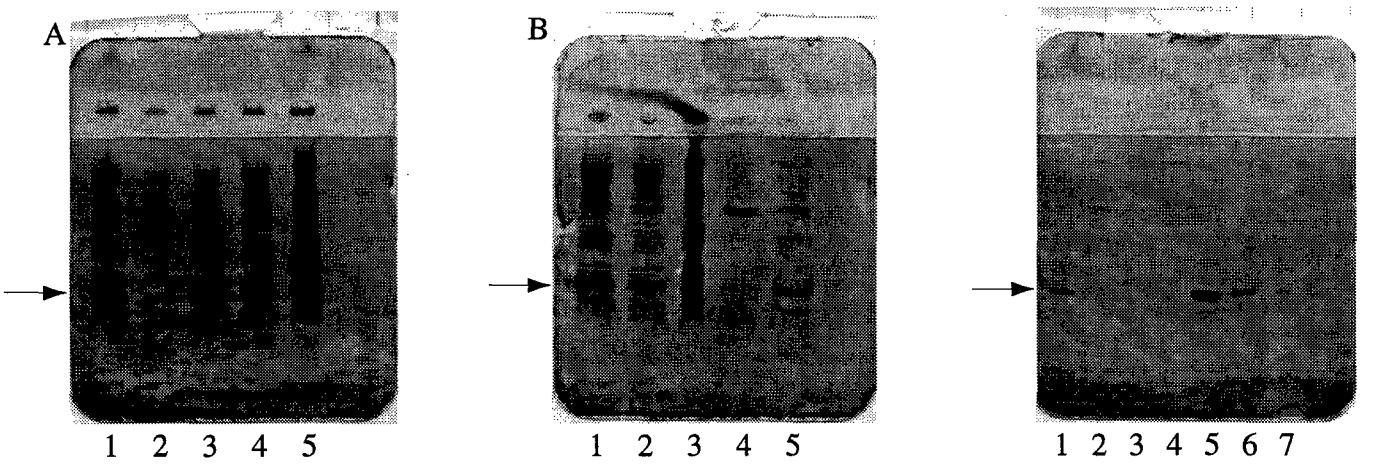
Appendices



Expression of hER[253-341]
2. uninduced cells
3. induced for 1.2 h
4. induced for 2.5 h
5. induced for 3.5 h
(1. same as 5 from a different experiment)

Fractionation of hER[253-341]
1. total protein
2. soluble fraction
3. soluble fraction after second extraction (not normalized)
4. insoluble fraction after urea extraction
5. urea-extracted fraction

Purification of hER[253-341]
1. urea-extracted proteins applied to an affinity column
2-4. washes
5-7. elution fractions

Figure 1. Expression (A), fractionation (B) and chromatography purification (C) of hER[253-351] shown on SDS-PAGE. The position of the hER fragment is indicated with an arrow.

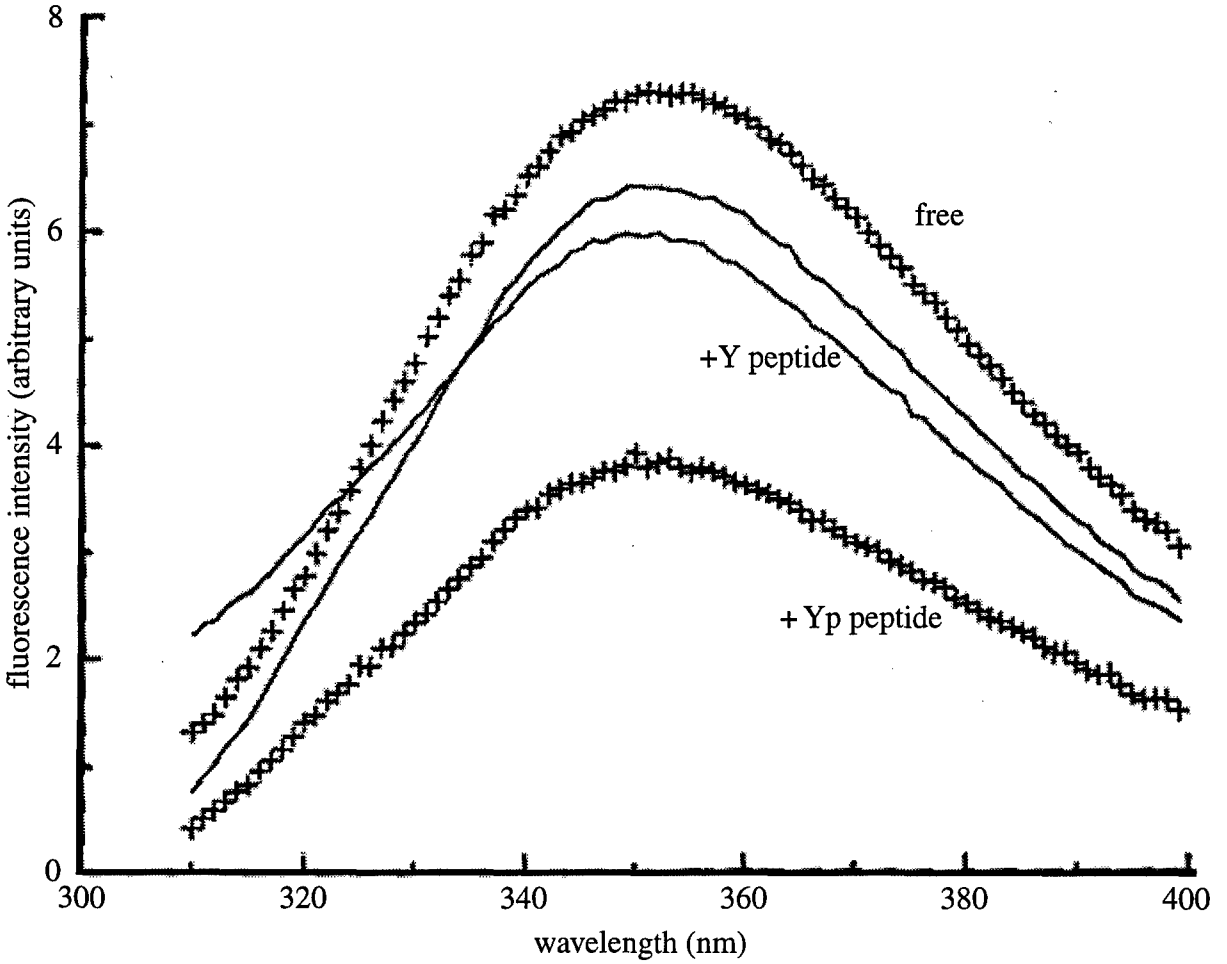


Figure 2. Fluorescence emission spectra of hER[253-341]. Spectra of the free protein (free), the protein with the phosphopeptide (+Yp peptide) and the protein with a control peptide (+Y peptide; two traces) are shown.

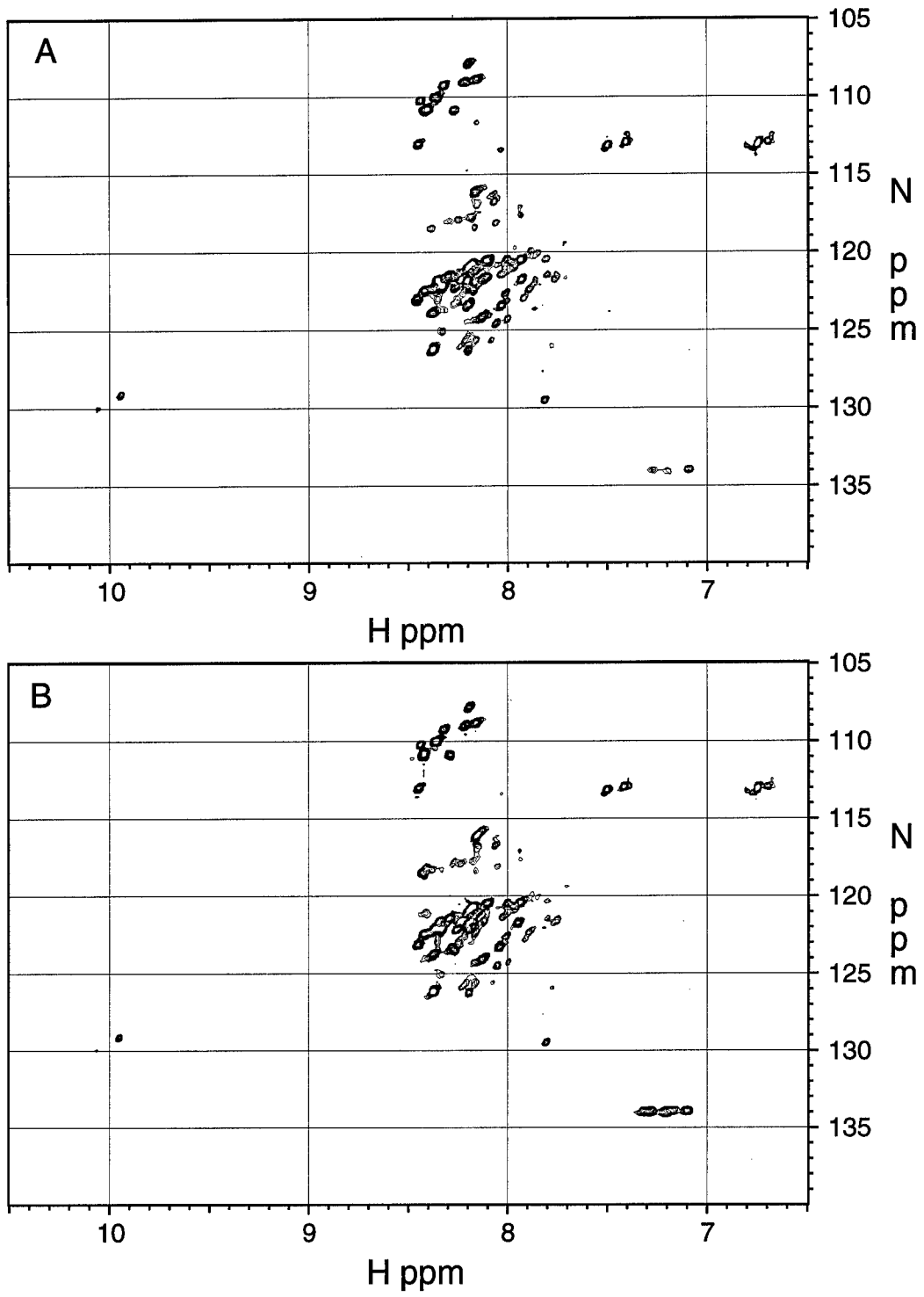


Figure 3. ^{1}H, ^{15}N HSQC spectra of free [^{15}N]-hER[253-341] (A) and [^{15}N]-hER[253-341] in the presence of the phosphopeptide. The protein and peptide concentrations were approximately 0.1 and 0.3 mM, respectively.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

Recd 8/16/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 9 August 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl